

University of Groningen

## Identification of Chloroacetaldehyde Dehydrogenase Involved in 1,2-Dichloroethane Degradation

Ploeg, Jan van der; Smidt, Marten P.; Landa, Andrew S.; Janssen, Dick B.

*Published in:*  
Biodegradation

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
1994

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Ploeg, J. V. D., Smidt, M. P., Landa, A. S., & Janssen, D. B. (1994). Identification of Chloroacetaldehyde Dehydrogenase Involved in 1,2-Dichloroethane Degradation. *Biodegradation*, 5(3).

### **Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### **Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

## Identification of Chloroacetaldehyde Dehydrogenase Involved in 1,2-Dichloroethane Degradation

JAN VAN DER PLOEG, MARTEN P. SMIDT, ANDREW S. LANDA, AND DICK B. JANSSEN\*

Department of Biochemistry, University of Groningen, 9747 AG Groningen, The Netherlands

Received 2 August 1993/Accepted 15 February 1994

The degradation of 1,2-dichloroethane and 2-chloroethanol by *Xanthobacter autotrophicus* GJ10 proceeds via chloroacetaldehyde, a reactive and potentially toxic intermediate. The organism produced at least three different aldehyde dehydrogenases, of which one is plasmid encoded. Two mutants of strain GJ10, designated GJ10M30 and GJ10M41, could no longer grow on 2-chloroethanol and were found to lack the NAD-dependent aldehyde dehydrogenase that is the predominant protein in wild-type cells growing on 2-chloroethanol. Mutant GJ10M30, selected on the basis of its resistance to 1,2-dibromoethane, also had lost haloalkane dehalogenase activity and Hg<sup>2+</sup> resistance, indicating plasmid loss. From a gene bank of strain GJ10, different clones that complemented one of these mutants were isolated. In both transconjugants, the aldehyde dehydrogenase that was absent in the mutants was overexpressed. The enzyme was purified and was a tetrameric protein of 55-kDa subunits. The substrate range was rather broad, with the highest activity measured for acetaldehyde. The *K<sub>m</sub>* value for chloroacetaldehyde was 160  $\mu$ M, higher than those for other aldehydes tested. It is concluded that the ability of GJ10 to grow with 2-chloroethanol is due to the high expression level of an aldehyde dehydrogenase with a rather low activity for chloroacetaldehyde.

*Xanthobacter autotrophicus* GJ10 can utilize 1,2-dichloroethane (1,2-DCE) and a variety of other chlorinated hydrocarbons as sole carbon and energy source (8). The degradation of 1,2-DCE proceeds via two dehalogenation reactions, catalyzed by different enzymes, and two oxidation steps (Fig. 1). The haloalkane dehalogenase, which is encoded on a large plasmid designated pXAU1 (20), hydrolyzes 1,2-DCE to 2-chloroethanol (11), which is oxidized by an aspecific PQQ-dependent alcohol dehydrogenase (6). Chloroacetaldehyde is oxidized further by a NAD-dependent aldehyde dehydrogenase (6) to chloroacetic acid, which is hydrolyzed by a haloacid dehalogenase (23). This degradation route has also been observed in *X. autotrophicus* GJ11 and strains of *Ancylobacter aquaticus* that utilize 1,2-DCE (22). The haloalkane dehalogenase nucleotide sequences in these strains were identical.

The ability to grow with 1,2-DCE has been attributed to the presence of the two hydrolytic dehalogenases (6). Several observations, however, suggest that there is also a special role for a plasmid-encoded aldehyde dehydrogenase, which is overexpressed at very high levels during growth on 2-chloroethanol. Loss of the plasmid impairs growth on 2-chloroethanol and 1,2-DCE (20). Halogenated aldehydes are toxic and mutagenic compounds (10, 13) and may be critical intermediates in the degradation pathway (11, 20). Many microorganisms able to grow with chloroacetic acid have been isolated, but only a few can also grow with 2-chloroethanol, although they utilize other alcohols. In addition, *X. autotrophicus* XD(pPJ66), which contains the cloned haloacid dehalogenase gene (23), can grow with ethanol and chloroacetic acid but not with 2-chloroethanol (5a). These observations suggest that an aldehyde dehydrogenase specifically adapted to the conversion of chloroacetaldehyde may be required for 2-chloroethanol conversion.

In this paper, we further describe the role and the properties of the plasmid-encoded chloroacetaldehyde dehydrogenase.

We report the cloning, purification, and characterization of the enzyme and show that the low affinity for chloroacetaldehyde may be the main reason for the high expression level.

### MATERIALS AND METHODS

**Organisms and growth conditions.** *X. autotrophicus* GJ10 has been described previously (6, 8) and was grown at 30°C in nutrient broth (Difco) or in a defined medium designated MMY (as in reference 8 but with 10 mg of yeast extract [Difco] per liter replacing the vitamins) containing a 5 mM concentration of a carbon source in closed flasks. *Escherichia coli* HB101 (1) was grown in LB medium at 30 or 37°C. For plates, 1.5% agar was added. Plasmids were maintained by the addition of tetracycline (12.5  $\mu$ g/liter) or kanamycin (50  $\mu$ g/liter).

*X. autotrophicus* GJ10M30 is a cured derivative of strain GJ10. It was isolated as a mutant resistant to 1,2-dibromoethane by spreading cells on plates containing 10 mM citrate and 10  $\mu$ l of 1,2-dibromoethane added on a paper filter disk on the edge of the plate. Resistant colonies which appeared after 1 week were restreaked and purified on MMY-citrate plates of the same composition. Strain GJ10M41 is a chloroacetaldehyde dehydrogenase-negative mutant of GJ10, but it still has haloalkane dehalogenase activity and thus contains the catabolic plasmid (6).

For purification of aldehyde dehydrogenase, GJ10M30 (pSS1) was grown aerobically in a 10-liter fermentor in MMY medium with 10 mM 2-chloroethanol as carbon source. Cells were grown for 4 days at 70% oxygen saturation. Additional 2-chloroethanol was added in amounts of 14 ml at intervals of about 7 h during the last 2 days of cultivation. The total amount of 2-chloroethanol added was 150 ml. After 2.5 days, 25 ml of salts solution (8), 500 mg of yeast extract, and 2 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O were added.

**Determination of Hg resistance.** Resistance to HgCl<sub>2</sub> was determined by plating cells in the late exponential phase of growth on nutrient broth. A hole was punched in the agar in the center of the plate, and 100  $\mu$ l of 1 mM HgCl<sub>2</sub> was added.

\* Corresponding author. Mailing address: Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Phone: (31) 50-634008. Fax: (31) 50-634165.

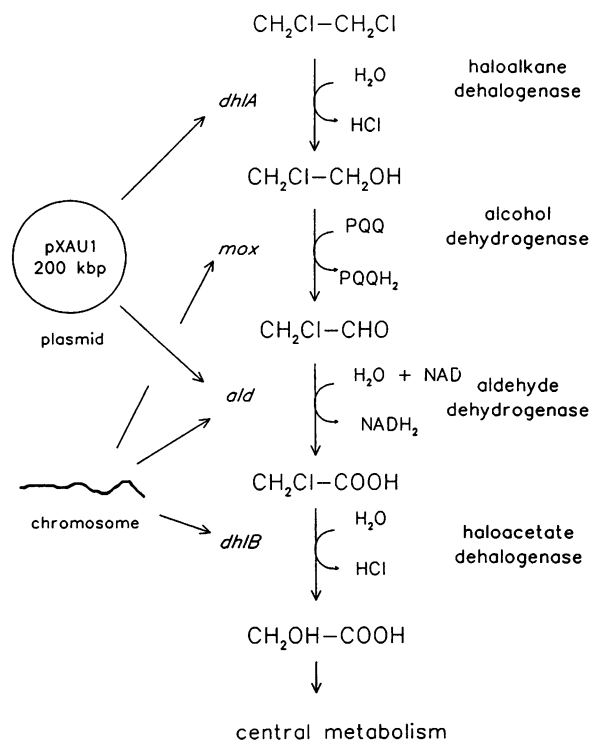


FIG. 1. Degradation pathway of 1,2-DCE by *X. autotrophicus* GJ10.

The zone of inhibition of growth was measured after 5 days of incubation at 30°C.

**Preparation of crude extracts and enzyme assays.** Crude extracts were prepared from cells grown to the late exponential phase, as described by Janssen et al. (8).

Haloalkane dehalogenase, PQQ-dependent methanol dehydrogenase, and haloacid dehalogenase activities were measured as described previously (8).

NAD-dependent aldehyde dehydrogenase activity was measured by monitoring NADH production at 340 nm and 30°C. The final assay mixture contained 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7 \cdot \text{H}_2\text{SO}_4$  (pH 8.75), 1 mM NAD, 1 mM dithiothreitol, 1 mM  $\beta$ -mercaptoethanol, and 10 mM substrate. Chloroacetaldehyde was used at a concentration of 2.5 mM unless stated otherwise. One unit of activity is defined as the amount catalyzing the formation of 1  $\mu\text{mol}$  of NADH  $\text{min}^{-1}$ . For determination of kinetic constants, aldehyde-containing solutions were heated at 100°C for 20 min before use and a range of concentrations was used.  $K_m$  and  $V_{\text{max}}$  values were obtained by using the Michaelis-Menten equation.

Protein concentrations were determined with Coomassie brilliant blue, using bovine serum albumin as a standard.

**Gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (23). Electroblothing to polyvinylidene difluoride membranes was done as described by Moos et al. (14).

Nondenaturing PAGE was done with 7.5% polyacrylamide gels (25). Gels were stained for NAD-dependent aldehyde dehydrogenase activity as described by Urakami and Komagata (21), except that  $\text{MgCl}_2$  and KCN were omitted from the staining solution. Staining was stopped by washing in distilled water and adding acetic acid to a final concentration of 10%.

**DNA manipulations and genetic procedures.** Plasmid isolation, restriction enzyme digestion, ligation, and transformation

were performed by standard protocols (17). Mobilization of pLAFR derivatives to *Xanthobacter* species was done according to the triparental mating procedure described previously (7), using pRK2013 as a helper plasmid delivering transfer functions (4).

Isolation of total DNA, Southern blotting, and hybridizations with a digoxigenin-labelled *dhIA* probe were as described previously (22).

**Purification of chloroacetaldehyde dehydrogenase.** Cells (25 g, dry weight) were harvested at  $11,000 \times g$  for 25 min and washed with TEMD buffer (10 mM Tris  $\cdot$   $\text{SO}_4$  [pH 7.5], 1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol, 1 mM dithiothreitol). To protect the enzyme against inactivation, all further operations were carried out at 4°C, and all buffers contained 1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol, and 1 mM dithiothreitol. Purification was followed by the measurement of aldehyde dehydrogenase activities, with butyraldehyde as the substrate. Cells were resuspended in 600 ml of TEMD buffer, divided, and disrupted in three 200-ml portions by sonication (20 min per portion in an Ultrasonics W-375 sonicator at 300-W output) under permanent cooling. Unbroken cells and debris were removed by centrifugation for 30 min at  $19,000 \times g$ .

The crude extract containing 22.6 mg of protein per ml was fractionated by stepwise addition of solid  $(\text{NH}_4)_2\text{SO}_4$  to 35 and 55% saturation. Precipitates were collected by centrifugation for 15 min at  $19,000 \times g$ . The 35 to 55% precipitate was suspended in TEMD buffer and dialyzed against this buffer for 36 h.

The dialyzed enzyme solution (200 ml) was applied to a DEAE-cellulose column (2.6 by 28 cm) equilibrated with TEMD buffer. The column was washed with 100 ml of buffer, and elution was carried out with a linear gradient of 0 to 0.5 M ammonium sulfate in buffer (total volume, 600 ml; flow rate, 60 ml/h; fraction volume, 10 ml). Activity was found at an ammonium sulfate concentration of 0.03 to 0.17 M. Active fractions were pooled and dialyzed against 5 mM potassium phosphate buffer (pH 7.5) for 60 h.

The dialysate (160 ml) was applied to a hydroxylapatite column (1.3 by 32 cm) equilibrated with 5 mM potassium phosphate buffer. A linear gradient of 5 to 100 mM potassium phosphate buffer (pH 7.5) was applied (total volume, 500 ml; flow rate, 30 ml/h; fraction volume, 10 ml). Activity eluted in two peaks: peak A passed the column without retention; peak B eluted at 50 to 95 mM potassium phosphate. Fractions of peak A contained the chloroacetaldehyde dehydrogenase activity as observed on activity-stained gels.

Peak A was dialyzed against TEMD buffer, and the dialysate was applied to a DEAE-cellulose column (2.6 by 28 cm) equilibrated with TEMD buffer. Elution was carried out with a 0 to 0.3 M linear gradient of ammonium sulfate in TEMD buffer (total volume, 500 ml; flow rate, 30 ml/h; fraction volume, 10 ml). Activity eluted at 0.02 to 0.11 M ammonium sulfate, and fractions were concentrated with a Diaflow PM30 filter.

The concentrated solution (40 ml) was applied to a Sephacryl S-200 column (3.7 by 68 cm) equilibrated with TEMD buffer (with 25 mM Tris) and then eluted with the same buffer (flow rate, 20 ml/h; fraction volume, 10 ml). Active fractions were pooled (150 ml) and concentrated with a Diaflow PM30 filter.

The molecular mass of the native enzyme was estimated by gel filtration on a Sephacryl S-300 column, as described above.

Determination of N-terminal protein sequences of purified and polyvinylidene difluoride-blotting protein was performed on a gas phase protein sequenator (model 477A; Applied

TABLE 1. NAD-dependent aldehyde dehydrogenase activities in crude extracts<sup>a</sup>

Strain	Growth substrate	Activity (mU/mg of protein)			
		AA	CAA	Butanal	Hexanal
GJ10	EtOH	639	252	119	42
	BuOH	63	78	116	84
GJ10M30	EtOH	37	20	ND	ND
	BuOH	32	34	90	84
GJ10M41	EtOH	21	20	ND	ND
	BuOH	35	34	68	52
GJ10M30(pPJ18)	EtOH	1,029	387	484	121
	BuOH	1,208	597	484	97
GJ10M30(pSS1)	EtOH	4,178	1,245	1,081	495
	BuOH	685	385	343	95
GJ10M30(pSS19)	EtOH	370	211	199	78
	BuOH	61	70	100	78
GJ10M41(pPJ19)	EtOH	ND	1,115	ND	ND
	BuOH	50	47	50	55

<sup>a</sup> Abbreviations: AA, acetaldehyde; CAA, chloroacetaldehyde; EtOH, ethanol; BuOH, butanol; ND, not determined.

Biosystems) by Eurosequence B.V. (Groningen, The Netherlands).

## RESULTS

**Characterization of mutants defective in 1,2-DCE degradation.** The roles of plasmid-encoded and chromosomally encoded aldehyde dehydrogenases in the utilization of alcohols by *X. autotrophicus* GJ10 were investigated by comparing the growth characteristics of a plasmid-less mutant with those of a strain with a chromosomal defect in aldehyde dehydrogenase expression. A derivative of strain GJ10 that lacks plasmid-encoded functions involved in 1,2-DCE degradation was isolated by selecting mutants resistant to 1,2-dibromoethane. One such derivative, designated strain GJ10M30, was unable to grow with 2-chloroethanol and 1,2-DCE. The toxicity of 1,2-dibromoethane was thus overcome by loss of the haloalkane dehalogenase, which is the first enzyme involved in its conversion to toxic products. The mutant was resistant to a 5 mM concentration of this compound in liquid culture, whereas 5  $\mu$ M completely inhibited growth of the wild type.

Hybridization experiments with a *dhlA* probe (22) showed that the *dhlA* gene had been deleted in GJ10M30 (results not shown). The inhibition of growth by HgCl<sub>2</sub> was tested. Strain GJ10M30 showed a zone of inhibition of 4.3 cm, whereas that of GJ10 was 1.7 cm. From these results, we concluded that GJ10M30 is missing the plasmid that harbors the haloalkane dehalogenase gene.

In extracts of cells of strain GJ10M30 grown on ethanol, no haloalkane dehalogenase activity could be detected, whereas the haloacid dehalogenase activity and PQQ-dependent methanol dehydrogenase levels were similar to those of the wild type (results not shown). The level of chloroacetaldehyde dehydrogenase in ethanol-grown cells of strain GJ10M30 was sevenfold lower than in the wild type (Table 1), indicating that this enzyme is affected in the wild type. Strain GJ10M30 was still able to grow on plates with methanol, ethanol, butanol, chloroacetic acid, and toluene.

A mutant with a chromosomal defect in aldehyde dehydrogenase expression, GJ10M41, has been described previously (6). This strain is unable to grow with 1,2-DCE and 2-chloroethanol because it lacks chloroacetaldehyde dehydrogenase activity, but it still expresses haloalkane dehalogenase and

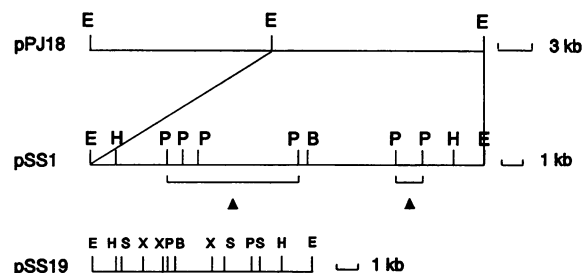


FIG. 2. Restriction site map of pPJ18, pSS1, and pSS19. Restriction sites: E, *EcoRI*; P, *PstI*; B, *BamHI*; H, *HindIII*; X, *XhoI*; S, *SalI*. The *PstI* fragments that are detected in pSS1 are indicated (▲).

HgCl<sub>2</sub> resistance, indicating that the catabolic plasmid is not deleted. Growth on ethanol was poor (doubling time of about 40 h), but growth with several other nonalcohol substrates was similar to that of GJ10 and GJ10M30.

These results show that GJ10M30 is no longer able to grow with chloroethanol due to the loss of plasmid pXAU1. Since growth with other alcohols is not affected, the plasmid-encoded enzyme is essential only for growth with 2-chloroethanol. Mutant GJ10M41 is still able to grow with longer-chain alcohols but not with ethanol or 2-chloroethanol. Since this mutant still contains the plasmid and the effect of its defect is more pronounced than that of plasmid loss, a chromosomal mutation must be responsible for this growth characteristic.

**Complementation of aldehyde dehydrogenase-negative mutants.** A gene bank of *X. autotrophicus* GJ10 in the broad-host-range cosmid vector pLAFR1 (7) was mated to both mutants, and transconjugants were screened for the ability to restore growth on 2-chloroethanol. Plasmids pPJ18 and pPJ19 were isolated for the ability to complement the mutations in GJ10M30 and GJ10M41, respectively. Plasmid pPJ18 could not complement GJ10M41, whereas pPJ19 could not complement GJ10M30. Therefore, both fragments of DNA are necessary for growth on 2-chloroethanol. A mutation in either one of the loci results in toxicity of 2-chloroethanol.

Strain GJ10M30(pPJ18) still could not grow with 1,2-DCE. Plasmid pPJ10, encoding *dhlA* on an insert of 29.5 kb (7), could not restore growth on 2-chloroethanol of strain GJ10M30. Thus, it can be concluded that the genes encoding haloalkane dehalogenase and aldehyde dehydrogenase are not closely linked. Complementation experiments indicated that resistance to HgCl<sub>2</sub> was not encoded on pPJ18 or pPJ10.

Plasmid pPJ18 contained two *EcoRI* inserts of 20 and 15 kb. Subcloning with *EcoRI* and *PstI* in the same vector showed that a 10.7-kb *PstI* fragment located on the 15-kb *EcoRI* fragment was sufficient for complementation of GJ10M30. A 4.5-kb *PstI* fragment was sufficient for complementation of GJ10M30, because two adjacent *PstI* fragments had been deleted (Fig. 2). Plasmid pPJ19 contained two *EcoRI* fragments of 2.3 and 1.8 kb.

**Analysis of aldehyde dehydrogenases present in the wild type and mutants.** Analysis of aldehyde dehydrogenase activities in strain GJ10 by activity assays (Table 1) and activity staining of nondenaturing protein gels (Fig. 3) indicated the presence of different aldehyde dehydrogenases. Butanol-grown cells of GJ10 had low activities of acetaldehyde and chloroacetaldehyde compared with cells grown on ethanol, whereas butyraldehyde activities were similar. Apparently, butanol and ethanol induce different aldehyde dehydrogenases. On the activity-stained gels (Fig. 3, lanes 1 and 2), one band with high mobility and three poorly separated bands with somewhat

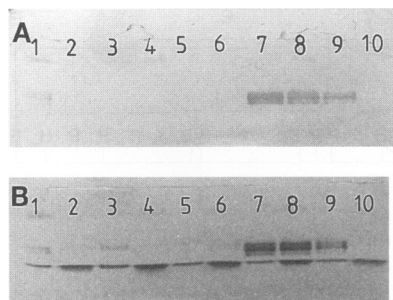


FIG. 3. Activity staining of NAD-dependent aldehyde dehydrogenases on polyacrylamide gels, with (A) acetaldehyde or (B) butanal as the substrate. Lanes: 1, GJ10 grown on ethanol; 2, GJ10 grown on butanol; 3, GJ10M30 grown on ethanol; 4, GJ10M30 grown on butanol; 5, GJ10M41 grown on ethanol; 6, GJ10M41 grown on butanol; 7, GJ10M30(pSS1) grown on ethanol; 8, GJ10M30(pSS1) grown on butanol; 9, GJ10M41(pPJ19) grown on ethanol; 10, GJ10M41(pPJ19) grown on butanol.

lower mobilities could be detected. The slowly migrating bands were absent when GJ10 was grown on butanol. In the butanal-stained gel, the fast-migrating band was present in both extracts, but it was more prominent in extracts from cells grown on butanol.

With GJ10M30 as well with GJ10M41, ethanol-grown cells had low acetaldehyde and chloroacetaldehyde dehydrogenase activities, and butanol-grown cells of the mutants had somewhat lower activities than the wild type. Probably, the aldehyde dehydrogenase preferentially induced by ethanol is no longer present in both mutants. The activity-stained gels showed that in both mutants the fast-migrating band was still present, whereas only one of the three slowly migrating bands was visible. For GJ10M30 grown on ethanol, this band was clearly visible, but in GJ10M41 it was barely visible. This may explain the difference observed between the growth of GJ10M30 and that of GJ10M41 on ethanol.

The complemented mutant GJ10M30(pPJ18) contained much higher aldehyde dehydrogenase activities than the wild-type strain for all substrates tested, independent of the substrate used for growth. Strain GJ10M30(pSS1) also overexpressed aldehyde dehydrogenase when grown on ethanol or butanol (Table 1; Fig. 3). Overexpression of aldehyde dehydrogenase in GJ10M30(pSS19) was lower, with activity being similar to that of the wild-type strain.

Strain GJ10M41(pPJ19) also had higher chloroacetaldehyde dehydrogenase activities but only when grown on ethanol. When grown on butanol, there was no major difference in activity between GJ10M41(pPJ19) and the wild-type strain (Table 1; Fig. 3).

The results indicate that at least three aldehyde dehydrogenases are present in GJ10. One of these forms one or more of the slowly migrating bands, has relatively high activities with acetaldehyde and chloroacetaldehyde, and has very low activity in the mutants. We will refer to this as chloroacetaldehyde dehydrogenase. Another is more active with longer-chain aldehydes and is still present in both mutants, enabling GJ10M30 and GJ10M41 to grow with nonchlorinated alcohols. A less pronounced, slowly migrating band is detected on butanal-stained gels with extracts of GJ10 and GJ10M30, but it is very faint in GJ10M41, which may explain the poor growth of the latter strain on ethanol.

Extracts of ethanol-grown cells of the mutants and the complemented strains were analyzed by SDS-PAGE. The

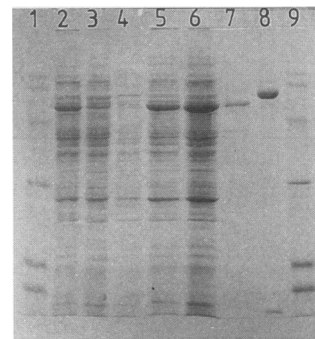


FIG. 4. SDS-PAGE of crude extracts and partially purified chloroacetaldehyde dehydrogenase. Lanes: 1, marker proteins; 2, GJ10; 3, GJ10M30; 4, GJ10M41; 5, GJ10M30(pPJ18); 6, GJ10M41(pPJ19); 7, gel-purified chloroacetaldehyde dehydrogenase; 8, purified methanol dehydrogenase (10); 9, marker proteins (ovotransferrin [78 kDa], albumin [66 kDa], ovalbumin [45 kDa], carbonic anhydrase [30 kDa], and myoglobin [17.2 kDa]).

protein band corresponding to the chloroacetaldehyde dehydrogenase activity from GJ10M30(pSS1) grown on ethanol (Fig. 3A, slot 7) was cut out from the gel and also subjected to PAGE (Fig. 4). The main difference between the various extracts is a predominant band of 55 kDa which is absent in the mutants but present in the wild type and complemented mutants. The mobility of this band is the same as that of the purified chloroacetaldehyde dehydrogenase. In GJ10M30(pPJ18) and GJ10M41(pPJ19), this protein is overexpressed.

**Purification of chloroacetaldehyde dehydrogenase.** Chloroacetaldehyde dehydrogenase was cut from a native gel, subjected to SDS-PAGE, and electroblotted to a polyvinylidene difluoride membrane. The N-terminal sequence of the blotted protein was determined to be Met-Asn-Lys/Pro-Pro-Glu/Ala-Ile-Ala-Ile-Thr-Lys.

NAD-dependent chloroacetaldehyde dehydrogenase was purified from strain GJ10M30(pSS1) as described in Materials and Methods (Table 2; Fig. 5). During hydroxylapatite chromatography, two peaks of activity eluted (Fig. 6A). Samples of the two peaks were subjected to nondenaturing gel electrophoresis (Fig. 6B). The first peak, which eluted without retention, had the same electrophoretic mobility as a slower-moving band in crude extracts of GJ10 and was free of aldehyde dehydrogenase of a different mobility. Therefore, this peak was chosen to further characterize the enzyme.

The molecular mass of the denatured protein was estimated to be 55 kDa by SDS-PAGE. When the enzyme was subjected to gel filtration, two peaks were detected, presumably the

TABLE 2. Purification of chloroacetaldehyde dehydrogenase

Step	Total protein (mg)	Total activity (U) <sup>a</sup>	Sp act (U/mg)
Crude extract	11,300	1,913	0.17
Ammonium sulfate	5,350	1,173	0.22
DEAE-cellulose I	4,680	1,320	0.28
Hydroxylapatite			
Peak A	1,510	770	0.51
Peak B	1,120	414	0.37
DEAE-cellulose II	1,400	438	0.31
Sephacryl S-200	450	453	1.01

<sup>a</sup> Activity was measured with butyraldehyde as the substrate.

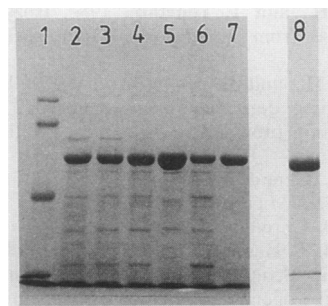


FIG. 5. SDS-PAGE of chloroacetaldehyde dehydrogenase-containing fractions during purification. Lanes: 1, marker proteins (ovo-transferrin [78 kDa], albumin [66 kDa], ovalbumin [45 kDa], and carbonic anhydrase [30 kDa]); 2, crude extract of GJ10M30(pSS1); 3,  $(\text{NH}_4)_2\text{SO}_4$  fractionation; 4, DEAE-cellulose I; 5, void volume of hydroxylapatite; 6, eluate of hydroxylapatite; 7, DEAE-cellulose II; 8, Sephacryl S-200.

monomer and the native enzyme. From gel filtration chromatography, we concluded that the native enzyme is a tetrameric protein with a molecular weight of 220,000.

The N-terminal sequence was determined (Met-Asn-Lys-Pro-Glu-Ile-Ala-Ile-Thr-Lys-Gln) and was the same as described above for the protein isolated from the native gel.

Activities of the enzyme with different substrates and some  $K_m$  values were determined (Table 3). Activity was highest with acetaldehyde, with a  $k_{\text{cat}}$  value of  $4.7 \text{ s}^{-1}$  for the tetrameric enzyme molecule. The  $K_m$  value for chloroacetaldehyde was twofold higher than those for acetaldehyde and propionaldehyde. Low activities were detected with formaldehyde, benzaldehyde, and some other aldehydes.

## DISCUSSION

In this paper, we describe the properties of a chloroacetaldehyde dehydrogenase of *X. autotrophicus* GJ10 that is re-

TABLE 3. Substrate specificity of chloroacetaldehyde dehydrogenase<sup>a</sup>

Substrate	Relative activity (%)	$V_{\text{max}}$ (U/mg)	$K_m$ ( $\mu\text{M}$ )
Formaldehyde	9	ND <sup>b</sup>	ND
Acetaldehyde	100	1.291	$90 \pm 14$
Propionaldehyde	22	0.284	$62 \pm 9$
Butyraldehyde	80	1.031	$75 \pm 9$
Chloroacetaldehyde	50	0.650	$160 \pm 40$
Benzaldehyde	16	ND	ND
(DL)-Glyceraldehyde	17	ND	ND
Propionaldehyde	48	ND	ND
Glutaraldehyde	49	ND	ND

<sup>a</sup> The rates of NADH production with the different substrates are expressed as percentages of the rate observed with acetaldehyde (1.28 U/mg of protein). All substrates were used at 10 mM.

<sup>b</sup> ND, not determined.

quired for 2-chloroethanol utilization. The enzyme is plasmid encoded and possesses haloalkane dehalogenase activity and  $\text{Hg}^{2+}$  resistance, as described by Tardif et al. (20), who showed the presence of a 200-kb plasmid in strain GJ10. Our cured derivative strain GJ10M30 was selected on the basis of resistance to the suicide substrate 1,2-dibromoethane and appears very similar to the cured derivative TG129 (20).

In both strains, chloroacetaldehyde dehydrogenase activity was reduced but not completely absent. This indicates the presence of a chromosomally encoded acetaldehyde dehydrogenase with some activity for chloroacetaldehyde. In contrast to strain GJ10M30, mutant GJ10M41 is not able to grow on ethanol. Thus, the plasmid-encoded aldehyde dehydrogenase gene required for chloroacetaldehyde metabolism and cloned on the pPJ18 plasmid is required but not sufficient for growth on ethanol and 2-chloroethanol. In mutant GJ10M41, regulatory genes involved in the expression of the plasmid-encoded chloroacetaldehyde dehydrogenase appear to be affected.

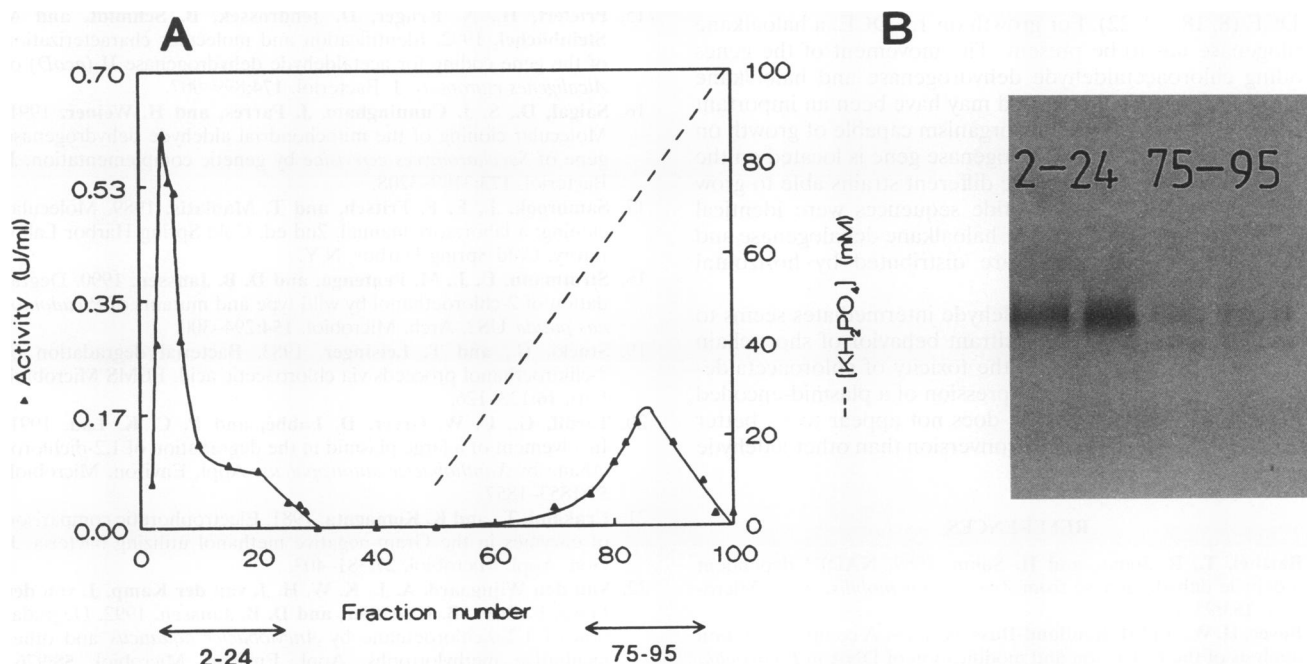


FIG. 6. (A) Separation of aldehyde dehydrogenases on hydroxylapatite. (B) Activity staining of active fractions.

Probably, the mutation affects formation of both plasmid-encoded chloroacetaldehyde dehydrogenase and chromosomally encoded acetaldehyde dehydrogenase, which allows growth of the cured mutant GJ10M30 on ethanol. The growth of the wild type and mutants on longer-chain alcohols can be explained by the presence of a third, chromosomally encoded aldehyde dehydrogenase with activity for long-chain aldehydes. This enzyme is not affected in either mutant. Different plasmid and chromosomally encoded aldehyde dehydrogenases have also been observed in *Pseudomonas oleovorans* (12). More than one enzyme has also been detected in several other bacterial strains (9, 24).

The enzyme was a tetrameric protein of molecular mass 55 kDa, which is similar to essentially all NAD-dependent aldehyde dehydrogenases that have been purified or sequenced (1, 12, 15, 16). The N-terminal sequence of the purified protein from GJ10 had 45% identity (5 of 11 amino acids) with the N terminus of aldehyde dehydrogenase II from *Alcaligenes eutrophus*, which is homologous to many other NAD-dependent aldehyde dehydrogenases (15). This indicates that the enzyme responsible for chloroacetaldehyde dehydrogenase is not a protein that is completely different from known aldehyde dehydrogenases but rather is a variant of a common class of enzymes.

Aldehydes are known to be toxic and mutagenic substances (10, 13). It is therefore likely to be important for the cell to keep the concentration as low as possible. The apparent  $K_m$  of chloroacetaldehyde dehydrogenase from strain GJ10 for chloroacetaldehyde (160  $\mu$ M) was higher than the  $K_m$  of the enzyme from strain US2 (27  $\mu$ M [18]). Aldehyde dehydrogenase purified from horse liver has a  $K_m$  value for chloroacetaldehyde of 30  $\mu$ M, although the enzyme is not involved in 2-chloroethanol degradation (3). It seems that the affinity of the purified enzyme for chloroacetaldehyde does not determine the ability to grow with 2-chloroethanol. Instead, the high expression level of the protein in GJ10 may be needed to prevent accumulation of toxic concentrations of chloroacetaldehyde.

It is relatively easy to isolate microorganisms able to grow with chloroacetic acid (5, our unpublished results), but only a few cultures are known to grow also with 2-chloroethanol or 1,2-DCE (8, 18, 19, 22). For growth on 1,2-DCE, a haloalkane dehalogenase has to be present. The movement of the genes encoding chloroacetaldehyde dehydrogenase and haloalkane dehalogenase to a single plasmid may have been an important step during the evolution of an organism capable of growth on 1,2-DCE. The haloalkane dehalogenase gene is located on the same restriction fragment in five different strains able to grow with 1,2-DCE, and the nucleotide sequences were identical (22). This indicates that both the haloalkane dehalogenase and the aldehyde dehydrogenase are distributed by horizontal transmission.

The accumulation of toxic aldehyde intermediates seems to be an important cause of recalcitrant behavior of short-chain halogenated alkanes. In GJ10, the toxicity of chloroacetaldehyde is overcome by the high expression of a plasmid-encoded aldehyde dehydrogenase, which does not appear to be better adapted to chloroacetaldehyde conversion than other aldehyde dehydrogenases.

#### REFERENCES

- Barthel, T., R. Jonas, and H. Sahm. 1989. NADP<sup>+</sup>-dependent aldehyde dehydrogenase from *Zymomonas mobilis*. Arch. Microbiol. 153:95–100.
- Boyer, H. W., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459–472.
- Eckfeldt, J. H., and T. Yonetani. 1982. Isozymes of aldehyde dehydrogenase from horse liver. Methods Enzymol. 89:474–479.
- Figurski, D. H., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA 76:1648–1652.
- Hardman, D. J., and J. H. Slater. 1981. Dehalogenases in soil bacteria. J. Gen. Microbiol. 123:117–128.
- Janssen, D. B. Unpublished data.
- Janssen, D. B., S. Keuning, and B. Witholt. 1987. Involvement of a quinoprotein alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase in 2-chloroethanol metabolism in *Xanthobacter autotrophicus* GJ10. J. Gen. Microbiol. 133:85–92.
- Janssen, D. B., F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra, and B. Witholt. 1989. Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. J. Bacteriol. 171:6791–6799.
- Janssen, D. B., A. Scheper, L. Dijkhuizen, and B. Witholt. 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. 49:673–677.
- Jendrossek, D., A. Steinbüchel, and H. G. Schlegel. 1987. Three different proteins exhibiting NAD-dependent acetaldehyde dehydrogenase activity from *Alcaligenes eutrophus*. Eur. J. Biochem. 167:541–548.
- Johnson, M. K. 1967. Metabolism of chloroethanol in the rat. Biochem. Pharmacol. 16:185–199.
- Keuning, S., D. B. Janssen, and B. Witholt. 1985. Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. 163:635–639.
- Kok, M., R. Oldenhuis, M. P. G. van der Linden, C. H. C. Meulenbergh, J. Kingma, and B. Witholt. 1989. The *Pseudomonas oleovorans* *alkBAC* operon encodes two structurally related rubredoxins and an aldehyde dehydrogenase. J. Biol. Chem. 264:5442–5451.
- McCann, J., V. Simmon, D. Streitwieser, and B. N. Ames. 1975. Mutagenicity of chloroacetaldehyde, a possible metabolic product of 1,2-dichloroethane (ethylene dichloride), chloroethanol (ethylene chlorohydrine), vinyl chloride and cyclophosphamide. Proc. Natl. Acad. Sci. USA 72:3190–3193.
- Moos, M., Jr., N. Y. Nguyen, and T.-Y. Liu. 1988. Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. J. Biol. Chem. 163:6005–6008.
- Priefert, H., N. Krüger, D. Jendrossek, B. Schmidt, and A. Steinbüchel. 1992. Identification and molecular characterization of the gene coding for acetaldehyde dehydrogenase II (*acoD*) of *Alcaligenes eutrophus*. J. Bacteriol. 174:899–907.
- Saigal, D., S. J. Cunningham, J. Farrés, and H. Weiner. 1991. Molecular cloning of the mitochondrial aldehyde dehydrogenase gene of *Saccharomyces cerevisiae* by genetic complementation. J. Bacteriol. 173:3199–3208.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Strotmann, U. J., M. Pentenga, and D. B. Janssen. 1990. Degradation of 2-chloroethanol by wild type and mutants of *Pseudomonas putida* US2. Arch. Microbiol. 154:294–300.
- Stucki, G., and T. Leisinger. 1983. Bacterial degradation of 2-chloroethanol proceeds via chloroacetic acid. FEMS Microbiol. Lett. 16:123–126.
- Tardif, G., C. W. Greer, D. Labbé, and P. C. K. Lau. 1991. Involvement of a large plasmid in the degradation of 1,2-dichloroethane by *Xanthobacter autotrophicus*. Appl. Environ. Microbiol. 57:1853–1857.
- Urakami, T., and K. Komagata. 1981. Electrophoretic comparison of enzymes in the Gram-negative methanol utilizing bacteria. J. Gen. Appl. Microbiol. 27:381–403.
- Van den Wijngaard, A. J., K. W. H. J. van der Kamp, J. van der Ploeg, F. Pries, B. Kazemier, and D. B. Janssen. 1992. Degradation of 1,2-dichloroethane by *Ancylobacter aquaticus* and other facultative methylotrophs. Appl. Environ. Microbiol. 58:976–983.

23. **Van der Ploeg, J., G. van Hall, and D. B. Janssen.** 1991. Characterization of the haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 and sequencing of the *dhlB* gene. J. Bacteriol. **173**:7925–7933.
24. **Weaver, C. A., and M. E. Lidstrom.** 1985. Methanol dissimilation in *Xanthobacter* H4-14: activities, induction and comparison to *Pseudomonas* AM1 and *Paracoccus denitrificans*. J. Gen. Microbiol. **131**:2183–2197.
25. **Weightman, A. J., and J. H. Slater.** 1980. Selection of *Pseudomonas putida* strains with elevated dehalogenase activities by continuous culture growth on chlorinated alkanolic acids. J. Gen. Microbiol. **121**:187–193.